TRANSLATION STATEMENT

Study Title: Bacterial Reverse Mutation Test of

Study Number: SR09242

I, the undersigned, hereby certify that this is a true and accurate translation from the original Japanese final report into English, which was reviewed properly.

Safety Research Institute for Chemical Compounds Co., Ltd.

Translated by:

Koko Nagaoka — May 14, 2012

Koko Nagaoka, Study Director — Date

FINAL REPORT

(Translation)

Study Title: Bacterial Reverse Mutation Test of

Study Number: SR09242

Safety Research Institute for Chemical Compounds Co., Ltd.

STATEMENT

Study Title: Bacterial Reverse Mutation Test of

Study Number: SR09242

1. This study was conducted in compliance with GLP standards "On the Standard for the Test Facility Conducting Tests Concerning New Chemical Substances, etc.," Notification No. 1121003 of the Pharmaceutical and Food Safety Bureau (PFSB), Ministry of Health, Labour and Welfare, Japan (MHLW), November 21, 2003; No. 3 of the Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry, Japan (METI), November 17, 2003; and No. 031121004 of the Environmental Policy Bureau, Ministry of the Environment, Japan (MOE); and "Partial Amendment of 'On the Standard for the Test Facility Conducting Tests Concerning New Chemical Substances, etc.'," Notification No. 0704001 of the PFSB, MHLW, July 4, 2008; No. 2 of the Manufacturing Industries Bureau, METI, June 30, 2008; and No. 080704001 of the Environmental Policy Bureau, MOE. The test method used in this study was based on "On the Test Method Concerning New Chemical Substances, etc." (Notification No. 1121002 of the PFSB, MHLW, November 21, 2003; No. 2 of the Manufacturing Industries Bureau, METI, November 13, 2003; and No. 031121002 of the Environmental Policy Bureau, MOE); and "Partial Amendment of 'On the Test Method Concerning New Chemical Substances, etc.'" (Notification No. 1120001 of the PFSB, MHLW, November 20, 2006; No. 2 of the Manufacturing Industries Bureau, METI, November 13, 2006; and No. 061120001 of the Environmental Policy Bureau, MOE)
2. This study was conducted in compliance with the study protocol, and no environmental factors that could have affected the reliability of the study were found.

Safety Research Institute for Chemical Compounds Co., Ltd.

Name and seal affixed in the original	August 10, 2010
Koko Nagaoka, Study Director	Date

QUALITY ASSURANCE STATEMENT

Study Title: Bacterial Reverse Mutation Test of

Study Number: SR09242

This study was inspected by the Quality Assurance Unit of Safety Research Institute for Chemical Compounds Co., Ltd. as follows:

Phase of study inspection	Date of inspection	Date of report to Study Director	Date of report to Management
Study protocol	April 23, 2010	April 23, 2010	April 23, 2010
Study protocol amendment (No. 1)	June 4, 2010	June 4, 2010	June 7, 2010
Study protocol amendment (No. 2)	June 29, 2010	June 29, 2010	June 29, 2010
Receipt, labeling and storage of test substance	April 23, 2010	April 23, 2010	April 23, 2010
Preparation of test substance	May 19, 2010	May 19, 2010	May 19, 2010
Conduct of study	May 19, 2010	May 19, 2010	May 19, 2010
Observation and colony counting	May 21, 2010	May 21, 2010	May 21, 2010
Raw data	June 23, 2010	June 23, 2010	June 23, 2010
Final report (draft): Tables and Figures	June 23, 2010	June 23, 2010	June 23, 2010
Final report (draft): Text	June 23, 2010	June 23, 2010	June 23, 2010
Final report	August 10, 2010	August 10, 2010	August 10, 2010

1. This study was conducted in compliance with "On the Standard for the Test Facility Conducting Tests Concerning New Chemical Substances, etc.," Notification No. 1121003 of the Pharmaceutical and Food Safety Bureau (PFSB), Ministry of Health, Labour and Welfare, Japan (MHLW), November 21, 2003; No. 3 of the Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry, Japan (METI), November 17, 2003; No. 031121004 of the Environmental Policy Bureau, Ministry of the Environment, Japan (MOE); "Partial Amendment of 'On the Standard for the Test Facility Conducting Tests Concerning New Chemical Substances, etc.'," Notification No. 0704001 of the PFSB, MHLW, July 4, 2008; No. 2 of the Manufacturing Industries Bureau, METI, June 30, 2008; and No. 080704001 of the Environmental Policy Bureau, MOE; "On the Test Method Concerning New Chemical Substances, etc." (Notification No. 1121002 of the PFSB, MHLW, November 21, 2003; No. 2 of the Manufacturing Industries Bureau, METI, November 13, 2003; and No. 031121002 of the Environmental Policy Bureau, MOE); and "Partial Amendment of 'On the Test Method Concerning New Chemical Substances, etc.'" (Notification No. 1120001 of the PFSB, MHLW, November 20, 2006; No. 2 of the Manufacturing Industries Bureau, METI, November 13, 2006; and No. 061120001 of the Environmental Policy Bureau, MOE).

2. The Quality Assurance Unit has reviewed the final report and determined the following: this study was conducted in compliance with the study protocol, the methods and procedures of this study were accurately described in this report, and the results presented in this report accurately reflect the raw data generated during this study.

Safety Research Institute for Chemical Compounds Co., Ltd.

Name and seal affixed in the original	August 10, 2010
Taku Katano, QA Representative	Date

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Study title: Bacterial reverse mutation test of

Study number: SR09242

Purpose of the study: was investigated for the potential to induce genetic mutation in bacteria using *Salmonella typhimurium* and *Escherichia coli*.

Good Laboratory Practice standards and test guidelines

Good Laboratory Practice (GLP) standards:

"On the Standard for the Test Facility Conducting Tests Concerning New Chemical Substances, etc.," Notification No. 1121003 of the Pharmaceutical and Food Safety Bureau (PFSB), Ministry of Health, Labour and Welfare, Japan (MHLW), November 21, 2003; No. 3 of the Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry, Japan (METI), November 17, 2003; and No. 031121004 of the Environmental Policy Bureau, Ministry of the Environment, Japan (MOE)

"Partial Amendment of 'On the Standard for the Test Facility Conducting Tests Concerning New Chemical Substances, etc.'," Notification No. 0704001 of the PFSB, MHLW, July 4, 2008; No. 2 of the Manufacturing Industries Bureau, METI, June 30, 2008; and No. 080704001 of the Environmental Policy Bureau, MOE

Test guidelines: "On the Test Method Concerning New Chemical Substances, etc." (Notification No. 1121002 of the PFSB, MHLW, November 21, 2003; No. 2 of the Manufacturing Industries Bureau, METI, November 13, 2003; and No. 031121002 of the Environmental Policy Bureau, MOE)

"Partial Amendment of 'On the Test Method Concerning New Chemical Substances, etc.'" (Notification No. 1120001 of the PFSB, MHLW, November 20, 2006; No. 2 of the Manufacturing Industries Bureau, METI, November 13, 2006; and No. 061120001 of the Environmental Policy Bureau, MOE)

Study sponsor
Name:
Address:
Study monitor:

Test facility

Name:	Safety Research Institute for Chemical Compounds Co., Ltd.
Address:	363-24 Shin-ei, Kiyota-ku, Sapporo 004-0839, Japan
Management:	Masao Kiguchi

Study Director

Name:	Koko Nagaoka
Company and division:	Safety Research Division, Safety Research Institute for Chemical Compounds Co., Ltd.

Study personnel

Test substance management:	Shiho Kodama, Misako Ohkubo, Kohtaro Kawamura, and Takashi Sakakibara
Test operation:	Koko Nagaoka, Takashi Sakakibara, and Wataru Shimatani

Testing period

Study initiation:	April 23, 2010
Receipt of test substance:	January 18, 2010
Start of test operation:	May 19, 2010
Dose-finding test	
Initiation of subculture:	May 18, 2010
Treatment with test substance:	May 19, 2010
Colony counting:	May 21, 2010
Main test	
Initiation of subculture:	June 1, 2010
Treatment with test substance:	June 2, 2010
Colony counting:	June 4, 2010
Confirmatory test	
Initiation of subculture:	June 8, 2010
Treatment with test substance:	June 9, 2010
Colony counting:	June 11, 2010
Experiment completion:	June 11, 2010
Study completion:	August 10, 2010

SUMMARY

The potential of to induce genetic mutation in bacteria was evaluated by a reverse mutation test using *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 and *Escherichia coli* WP2*uvr*A. The test was conducted by the pre-incubation method, in the non-activated assay (in the absence of the S9 mix metabolic activation system) and activated assay (in the presence of the S9 mix metabolic activation system).

In the dose-finding test, the highest dose of the test substance was set at 5000 μg/plate, and a total of 7 doses (5 to 5000 μg/plate) were set in approximately threefold dilution series (6-step serial dilution) for each test strain in both the non-activated and activated assays. In the main test, the highest dose of the test substance was set at 5000 μg/plate, and a total of 7 doses (78.1 to 5000 μg/plate) were set in a twofold dilution series (6-step serial dilution) for each test strain in both the non-activated and activated assays. In addition, a confirmatory test was conducted using *S. typhimurium* TA98 by the non-activated assay at the same doses as in the main test.

The results of the dose-finding test were as follows: In the non-activated assay using *S. typhimurium* TA98, the average numbers of revertants at 150 to 1500 μg/plate increased and was twice or more than in the negative control group, which was not dose-related. The average number of revertants in the test substance groups was less than twice that in the negative control group in the other test strains both the non-activated and activated assays, and no dose-related increase in the number of revertants was noted. No inhibition of cell growth or precipitation of the test substance was detected in any test strains, either the non-activated and activated assays.

The results of the main and confirmatory tests were as follows: the average number of revertants in the test substance groups were less than twice that in the negative control group in each test strain both the non-activated and activated assays, and no dose-related increase in the number of revertants was noted. No inhibition of cell growth or precipitation of the test substance was detected in any test strains, under all test conditions used in these tests.

In the dose-finding, main, and confirmatory tests, all the average numbers of revertants in the negative control group of all test strains were within the control range based on the historical control data of the test facility. The average number of revertants in the positive control group clearly increased and was twice or more that in the negative control group. These results confirmed that each test strain had appropriate sensitivity to the mutagens.

On the basis of these results, the increase in the numbers of revertants in *S. typhimurium* TA98 in the non-activated assay in the dose-finding test was not dose-related, which showed no reproducibility; therefore, this was not considered to be positive reaction. In conclusion, is not mutagenic to the bacteria under the conditions of this test.

INTRODUCTION

The potential of to induce genetic mutation in bacteria was evaluated by a reverse mutation test using *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 and *Escherichia coli* WP2*uvr*A. The test was conducted by the pre-incubation method, in the non-activated assay (in the absence of the S9 mix metabolic activation system) and activated assay (in the presence of the S9 mix metabolic activation system).

MATERIALS AND METHODS

1. Test substance

Name:
Abbreviation:
CAS No.:

Molecular formula:
Structural formula:

Molecular weight:
Physicochemical properties:

Lot No.:

Supplier:	Study sponsor
Amount obtained:	2 containers (NET 1000 g and 1090 g, shared with related tests) Obtained as an aqueous solution (clear and colorless liquid) containing solid content 24.0%, impurities 0.85% (to the solid content), and Cl ion 35 ppm (Appendix 1-1).
Stability:	After completion of test operation, the analysis results of the test substance were obtained and stability was confirmed (Appendix 1-2).
Storage conditions:	Containers should be sealed to avoid moisture absorption. Direct sunlight is strictly prohibited (protected from light), and attention should be paid for water and high humidity

(measured temperature: 2 to 8 °C).

Storage location: Test substance storage room (January 18 [date received] to April 29, 2010) and mutagenicity test room (April 29 to June 9, 2010 [date of treatment with the test substance in the confirmatory test])

Caution in handling: Sufficient ventilation should be provided in the workplace. Proper protection equipment such as protection glasses and gloves are used. Generating dust should be avoided.

Remaining test substance: After completion of all test operation including that in the related tests, the remaining test substance was returned to the supplier.

2. Preparation of test substance

Preparation method: The test substance was dissolved in and diluted with Japanese Pharmacopoeia water for injection (Lot No. 8L88, Otsuka Pharmaceutical Factory, Inc.) to the prescribed concentrations. Calculations for the preparation was made on the basis that the purity of the test substance was 24.0% by the following procedure: For the dose-finding test, the 50 mg/mL preparation (value calculated from the purity: 208.33 mg/mL) was serially diluted with Japanese Pharmacopoeia water for injection in approximately threefold dilution series to prepare 15, 5, 1.5, 0.5, 0.15 and 0.05 mg/mL preparations. For the main and confirmatory tests, the 50 mg/mL preparation (value calculated from the purity: 208.33 mg/mL) was serially diluted with the vehicle in a twofold dilution series to prepare 25, 12.5, 6.25, 3.13, 1.56, and 0.781 mg/mL preparations.

Rationale for selection of vehicle:

Because the test substance is soluble in water.

Preparation frequency: The preparation was prepared before use, and used within 2.0 h in the dose-finding test, within 2.1 h in the main test, and within 0.7 h in the confirmatory test.

Caution in preparation: Prepared in a clean bench using protection glasses, protection mask, masks, gloves, and white lab coat to avoid inhalation and contact with eyes, skin and clothes.

Confirming for stability of preparations:

Preparations showed no visible reactivity (such as discoloration, generation of heat, and foaming) at preparation of test substance in the dose-finding, main, and confirmatory tests.

Disposal of the remaining preparations:

Remaining preparations were collected as industrial waste to be incinerated.

3. Negative control substance

Japanese Pharmacopoeia water for injection (Lot No. 8L88; Otsuka Pharmaceutical Factory, Inc.), which was the vehicle used to prepare the test substance, was used as the negative control substance without any further preparation.

4. Positive control substances and their preparation

The known mutagens shown in the table below were used as the positive control substances. These positive control substances were stored in a cool and dark place (set at 2 to 8 °C).

Positive control substances were prepared to the concentrations shown in the table below without correction for the content. The preparations were aliquoted and cryopreserved at -20°C or below, and used within 1.8 h after thawing. The preparations were used within 9 months after the day of preparation (expiration: one year after preparation). Remaining preparations of the positive control substance were collected as the industrial waste to be incinerated.

Positive control substance	Concentration	Preparation vehicle
2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (content: 98.3%) Lot No. SDJ4376 Wako Pure Chemical Industries, Ltd.	0.1 and 1 μg/mL	Dimethyl sulfoxide Lot No. WF032 Dojindo Laboratories
Sodium azide (purity: 99.8%) Lot No. SDH6348 Wako Pure Chemical Industries, Ltd.	5 μg/mL	Japanese Pharmacopoeia water for injection Lot No. 8L88 Otsuka Pharmaceutical Factory, Inc.
9-Aminoacridine hydrochloride hydrate (purity: 99.9%) Lot No. S32398-347 Sigma-Aldrich Corporation	800 μg/mL	Dimethyl sulfoxide Lot No. WF032 Dojindo Laboratories
2-Aminoanthracene (content: 97.2%) Lot No. TSP5974 Wako Pure Chemical Industries, Ltd.	5, 10, 20 and 100 μg/mL	Dimethyl sulfoxide Lot No. FW032 Dojindo Laboratories

5. Bacterial test strains

The test strains used in this test were *Salmonella typhimurium* (expressed as "*S.typhimurium*" hereinafter) TA100, TA1535, TA98, and TA1537 and *Escherichia coli* (expressed as "*E.coli*" hereinafter) WP2*uvr*A. These test strains were obtained from National Institute of Hygienic Sciences (current National Institute of Health Sciences) on October 18, 1991. The test strains were selected because they were widely accepted as the bacteria suitable for investigating the genotoxic potential of chemicals.

To 8 mL of the culture of each test strain, 0.7 mL of dimethyl sulfoxide (Lot No.

BT159; Dojindo Laboratories) was added. The culture was aliquoted and cryopreserved at -80°C or below. Using an aliquot of the culture of each test strain, characteristics of the test strain (amino acid-requirement, deep rough character [*rfa*], UV sensitivity, and drug resistance), and the number of revertants in the negative and positive control substance groups were checked, and the test strains in which these characteristics were normal were used in the test.

6. Culture medium

6.1. Culture medium for subculture

As the culture medium for subculture, nutrient broth medium (Oxoid Nutrient Broth No. 2; Lot No. 464616; Oxoid Ltd.) was prepared with Japanese Pharmacopoeia water for injection (Lot No. 8L88; Otsuka Pharmaceutical Factory, Inc.) to yield a concentration of 25 g/L. To the culture media of *S. typhimurium* TA98 and TA100, ampicillin sodium (Lot No. M8B0619; Nacalai Tesque, Inc.) was added to yield a concentration of 25 μg/mL before use.

6.2. Culture medium for the test (minimum glucose agar medium: expressed as "a plate" hereinafter)

The components in 1000 mL of the minimum glucose agar medium (Vitalmedia AMT-O; Lot No.DZLB2Q01; manufactured on February 26, 2010; Kyokuto Pharmaceutical Industrial Co., Ltd.) used as the culture medium for the test are shown in the following table.

Components in 1000 mL of culture medium for the test	
Magnesium sulfate heptahydrate	0.2 g
Citric acid monohydrate	2.0 g
Potassium phosphate dibasic	10.0 g
Ammonium phosphate monobasic	1.92 g
Sodium hydroxide	0.66 g
Glucose	20.0 g
Agar powder (Oxoid Agar No.1; Lot No. 1073337-02)	15.0 g

6.3. Top agar

Soft agar (shown as [A] in the table below) and amino acid solution (shown as [B] in the table below) with the components shown in the table below were prepared with Japanese Pharmacopoeia water for injection (Lot Nos. 8L88 and 9K88; Otsuka Pharmaceutical Factory, Inc.). Before use, (A) and (B) were mixed in a volume ratio of 10:1. Amino acid solution of L-histidine and D-biotin was applied to *S.typhimurium*, and that of L-tryptophan was applied to *E.coli*. These top agars were kept at 47°C until use.

Components of top agar	
(A) Soft agar	
Bacto™ Agar (Lot No. 9131109; Becton, Dickinson and Company)	0.6%
Sodium chloride (Lot No. 106P5602; Kanto Chemical Co., Ltd.)	0.5%
(B) Amino acid solution	
Solution of L-histidine and D-biotin	0.5 mmol/L each
(L-histidine; Lot No. WKK2889; Wako Pure Chemical Industries, Ltd.)	
(D-biotin; Lot Nos. PEN6828 and CDJ4769; Wako Pure Chemical Industries, Ltd.)	
or	
L-tryptophan solution	0.5 mmol/L
(L-tryptophan; Lot No. PEP6208; Wako Pure Chemical Industries, Ltd.)	

7. S9 mix

S9 mix was prepared with S9 (Lot No. RAA-609; manufactured on February 5, 2010; Kikkoman Corporation), Cofactor for S9 mix (Cofactor-I; Lot No. 999903; Oriental Yeast, Co., Ltd.) and Japanese Pharmacopoeia water for injection (Lot No. 8L88; Otsuka Pharmaceutical Factory, Inc.) before use.

S9 was cryopreserved at -80°C or below after purchase, and used within 4 months from the date of manufacture (expiration: 6 months after manufacture). The S9 was prepared from liver homogenates from Slc:SD rats (male, 7 weeks of age) to which phenobarbital and 5,6-benzoflabone had been intraperitoneally administered for enzyme induction. The components in 1 mL of S9 mix are shown in the following table:

Components in 1 mL of S9 mix		
S9	0.1	mL
Magnesium chloride	8	μmol
Potassium chloride	33	μmol
Glucose-6-phosphate	5	μmol
Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)	4	μmol
Nicotinamide adenine dinucleotide, reduced form (NADH)	4	μmol
Sodium-phosphate buffer (pH 7.4)	100	μmol

8. Test groups

8.1. Dose-finding test

The test was conducted by the non-activated assay (in the absence of metabolic activation system S9 mix) and by the activated assay (in the presence of metabolic activation system S9 mix) for each test strain.

For both of the non-activated and activated assays, the highest dose of the test substance was set at 5000 μg/plate (value calculated from the purity: 20833 μg/plate), and a total of 7 doses (5 to 5000 μg/plate: values calculated from the purity: 20.83 to 20833 μg/plate) were set in approximately threefold dilution series (6-step serial dilution). These test groups are shown in the following table:

Test series	Test group	Dose (μg/plate)	Number of plates
Non-activated assay	Test substance group	5	2
		15	2
		50	2
		150	2
		500	2
		1500	2
		5000	2
Activated assay	Test substance group	5	2
		15	2
		50	2
		150	2
		500	2
		1500	2
		5000	2

8.2 Main test and confirmatory test

These tests were conducted by the non-activated and activated assays for each test strain.

As the results of dose-finding test, though the number of revertants increased in *S. typhimurium* TA98 at 150 to 1500 μg/plate in the non-activated assay, inhibition of cell growth or precipitation of the test substance was not detected in any test strains in both the non-activated and activated assays; therefore, the highest concentration in the main test was set at 5000 μg/plate (value calculated from the purity: 20833 μg/plate), and a total of 7 doses (78.1 to 5000 μg/plate: values calculated from the purity: 325 to 20833 μg/plate) were set in twofold dilution series (6-step serial dilution).

A confirmatory test was conducted in *S. typhimurium* TA98 at the same doses as in the main test, in the non-activated assay.

These test groups in the main and confirmatory tests are shown in the following table:

Test series	Test group	Dose (μg/plate)	Number of plates
Non-activated assay	Test substance group	78.1	2
		156	2
		313	2
		625	2
		1250	2
		2500	2
		5000	2
Activated assay	Test substance group	78.1	2
		156	2
		313	2
		625	2
		1250	2
		2500	2
		5000	2

8.3. Negative and positive control groups

A negative control group (Japanese Pharmacopoeia water for injection) and a positive control group shown in the following table were set for each test strain in the dose-finding, main, and confirmatory tests.

Test strains	Positive control substance (dose: μg/plate)	
	Non-activated assay	Activated assay
S. typhimurium TA100	AF-2 (0.01)	2-AA (1)
S. typhimurium TA1535	NaN_3 (0.5)	2-AA (2)
E. coli WP2*uvr*A	AF-2 (0.01)	2-AA (10)
S. typhimurium TA98	AF-2 (0.1)	2-AA (0.5)
S. typhimurium TA1537	9-AA (80)	2-AA (2)

AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide

NaN_3: sodium azide 9-AA: 9-aminoacridine hydrochloride hydrate

2-AA: 2-aminoanthracene

8.4. Number and identification of plates

Each test strain was tested using 3 plates in the negative control group, 2 plates per dose in the test substance groups, and 2 plates in the positive control group. For identification, a label indicating the study number and the test group was attached to each plate.

9. Test method

9.1. Subculture of test strains

Each thawed test strain suspension (12 μL) was inoculated into an L-shaped tube (capacity approximately 40 mL) containing 12 mL of the culture medium for subculture (nutrient broth medium). The mixture in the L-shaped tube was cooled with ice until the start of incubation, for 7.5 h in the dose-finding test, 7.4 h in the main test, and 7.0 h in the confirmatory test. Then, the mixture was incubated for 10 h in a shaking water bath (Personal-11・EX, Taitec Corporation) set at 37°C, 40 mm in stroke, and at a rate 100 times/min. At the termination of incubation, OD_{660nm} of the culture obtained was measured using a spectrophotometer (mini photo 518, Taitec Corporation) to calculate the number of viable cells by the correlation equation between the number of viable cells and OD_{660nm}. A culture in which the number of viable cells was above 1×10^9cells/mL was used in the test.

The numbers of viable cells (calculated value) in the culture of each test strain were as follows:

Test strain	Number of viable cells (calculated value) ($\times 10^9$ cells/mL)		
	Dose-finding test	Main test	Confirmatory test
S. typhimurium TA100	2.60	2.52	–
S. typhimurium TA1535	3.20	3.20	–
E. coli WP2*uvr*A	4.42	4.29	–
S. typhimurium TA98	2.82	2.82	2.63
S. typhimurium TA1537	1.37	1.34	–

9.2. Treatment with the test and control preparations

The pre-incubation method was used for treatment with the test and control preparations. To a polyethylene tube (capacity of 5 mL) with a cap containing 0.1 mL of the test or control preparation, 0.5 mL of 0.1 mol/L sodium-phosphate buffer (pH 7.4) was added and mixed for the non-activated assay, and 0.5 mL of S9 mix was added and mixed for the activated assay. To these mixtures, 0.1 mL of each culture was added, which were incubated (pre-incubation) for 20 min in a shaking water bath (Personal-11・EX, Taitec Corporation) set at 37°C, 40 mm in stroke, and at a rate 100 times/min. After the termination of pre-incubation, 2 mL of top agar containing 0.05 mmol/L L-histidine and 0.05 mmol/L D-biotin was added to the mixtures of *S.typhimurium*, and 2 mL of top agar containing 0.05 mmol/L L-tryptophan was added to the mixtures of *E.coli*, which were mixed and spread over each plate. After the top agar was solidified on a flat place, the plates were placed in an incubator (MIR-262, SANYO Electric Co., Ltd.) set at 37°C and incubated for 49 h.

In each of the dose-finding, main, and confirmatory tests, a sterility test was performed to check bacterial contamination in the test preparation of the highest concentration and in the S9 mix used in the test.

9.3. Observation

The plates in the negative control group, test substance groups and positive control group of each test strain were observed with a stereoscopic microscope (SZ6045TR, Olympus Optical Co., Ltd.) for inhibition of cell growth, and the plates in the test substance groups were macroscopically checked for precipitation of the test substance. Then, revertants in each plate in the negative control group, test substance groups, and positive control group of each test strain were counted using a colony analyzer (CA-11D, System Science Co., Ltd.).

Evaluation of inhibition of cell growth was performed according to the following criteria, and an evaluation result scored 1 or more was considered to be inhibition of cell growth.

0: No inhibition of cell growth

Small background colonies (visible with a magnification approximately × 50) are observed all over the plate, and no difference is noted when compared with those in the negative control group.

1: Slight inhibition of cell growth

Density of background colonies decreases and the size of each colony increases when compared with those in the negative control group.

2: Moderate inhibition of cell growth

Protruding and large revertants and small-plane background colonies are observed on the same plate.

3: Severe inhibition of cell growth

Background colonies grow to the sizes equivalent to those of the revertants, and they are not distinguishable.

4: No survivors are observed.

9.4. Calculation of observation results

The average number of revertants was calculated for the negative control group, test substance groups (for each dose), and positive control group of each test strain.

10. Evaluation of test results

10.1. Confirmation of sensitivity of test strains

A test strain was judged to have appropriate sensitivity when the average number of revertants in the negative control group of the test strain was within the range of control values based on the historical control data of the test facility, and the value in the positive control group of the test strain was twice or more that in the negative control group.

10.2. Evaluation criteria of test results

The test substance was judged to be positive for potential to induce genetic mutation when the average number of revertants in the test substance groups was twice or more that in the negative control group, and reproducibility in the dose-related increase in the number of revertants was demonstrated, in at least one test strain. No statistical analyses were applied for evaluation of the test results.

10.3. Calculation of specific activity in mutagenicity

As positive results were not obtained in the test substance groups, specific activity in mutagenicity was not calculated in this test.

RESULTS

The results of the dose-finding test are shown in Tables 1 and 2, those in the main test in Tables 3 and 4, and those in the confirmatory test in Table 5. The dose-response curves obtained from the doses of the test substance and the numbers of revertants in the dose-finding, main, and confirmatory tests are shown in Figures 1-1 to 5-2.

The results of the dose-finding test (5 to 5000 μg/plate) were as follows: In *S. typhimurium* TA98 in the non-activated assay, the average numbers of revertants at 150 to 1500 μg/plate increased and was twice or more than in the negative control group, which was not dose-related. The average number of revertants in the test substance groups was less than twice that in the negative control group in the other test strains both the non-activated and activated assays, and no dose-related increase in the number of revertants was noted. No inhibition of cell growth or precipitation of the test substance was detected in any test strains, either the non-activated and activated assays.

The results of the main test (78.1 to 5000 μg/plate) were as follows: the average number

of revertants in the test substance groups were less than twice that in the negative control group in each test strain both the non-activated and activated assays, and no dose-related increase in the number of revertants was noted. No inhibition of cell growth or precipitation of the test substance was detected in any test strains, either the non-activated and activated assays.

As the increase in the number of revertants in *S. typhimurium* TA98 in the non-activated assay was not reproducible between the dose-finding test and the main test, a confirmatory test was conducted by the non-activated assay in *S. typhimurium* TA98 at the same doses as in the main test to assess reproducibility. As the results, the average number of revertants in the test substance groups was less than twice that in the negative control group, and no dose-related increase was noted, and no inhibition of cell growth or precipitation of the test substance was detected.

In the dose-finding, main, and confirmatory tests, all the average numbers of revertants in the negative control group of all test strains were within the control range based on the historical control data (Appendix 2) of the test facility. The average number of revertants in the positive control group of each test strain clearly increased, and was twice or more that in the negative control group in all test strains.

In the sterility tests in the dose-finding, main, and confirmatory tests, no bacterial contamination was detected in the test preparation of the highest dose or in the S9 mix.

DISCUSSION

The potential of to induce genetic mutation in bacteria was evaluated by a reverse mutation test using *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 and *Escherichia coli* WP2*uvr*A.

In the dose-finding test, the highest dose of the test substance was set at 5000 μg/plate, and a total of 7 doses were set in approximately threefold dilution series (6-step serial dilution) for each test strain in both the non-activated and activated assays. Based on the results of the dose-finding test, the highest dose of the test substance in the main test was set at 5000 μg/plate, and a total of 7 doses were set in a twofold dilution series (6-step serial dilution) for each test strain in both the non-activated and activated assays. In addition, a confirmatory test was conducted in *S. typhimurium* TA98 in the non-activated assay at the same doses as in the main test.

The results were as follows: In the non-activated assay using *S. typhimurium* TA98 in the dose-finding test, the average numbers of revertants at the intermediate to high doses increased and was twice or more than in the negative control group, which was not dose-related. The increase in the number of revertants was not detected in *S. typhimurium* TA98 in the non-activated assay in the main or confirmatory test. Thus, the increase in the numbers of revertants in *S. typhimurium* TA98 in the non-activated assay in the dose-finding test was not dose-related, and showed no reproducibility; therefore, this was not considered to be positive reaction. The increase in the number of revertants

in the dose-finding test was considered to be incidental change due to the low values in the negative control group, which were at the lowest level of the historical control data of the test facility.

In the other 4 test strains, the average number of revertants in the test substance groups was less than twice that in the negative control group of all test strains in the activated and non-activated assays, and no dose-related increase in the number of revertants was noted.

In the dose-finding, main, and confirmatory tests, all the average numbers of revertants in the negative control group of all test strains were within the control range based on the historical control data of the test facility. The average number of revertants in the positive control group clearly increased and was twice or more that in the negative control group. These results confirmed that each test strain had appropriate sensitivity to the mutagens.

In conclusion, is not mutagenic to the bacteria under the conditions of this test.

ENVIRONMENTAL FACTORS THAT MIGHT HAVE AFFECTED THE RELIABILITY OF THE TEST RESULTS

No environmental factors that might have affected the reliability of the test results were found.

STORAGE OF DOCUMENTS

The following documents will be stored in the archives of Safety Research Institute for Chemical Compounds Co., Ltd. for 10 years after the study completion, and their storage thereafter will be decided by an agreement with the study sponsor.

1.1. Study protocol and study protocol amendment
1.2. Raw data and other documents
1.3. Final report

NAME AND SEAL OF STUDY DIRECTOR

Name and seal affixed in the original	August 10, 2010
Koko Nagaoka, Study Director	Date

Table 1 Reverse mutation test of in *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 and *Escherichia coli* WP2*uvr* A without metabolic activation (dose-finding test) (SR09242)

Compound	Concentration (μg/plate)	Revertants per plate (Mean)				
		S9 (−)				
		TA100	TA1535	WP2*uvr* A	TA98	TA1537
Control [a]		116 , 111 110 (112)	12 , 13 10 (12)	12 , 11 19 (14)	12 , 8 12 (11)	12 , 9 11 (11)
	5	96 , 127 (112)	13 , 9 (11)	17 , 17 (17)	23 , 19 (21)	5 , 12 (9)
	15	128 , 119 (124)	10 , 10 (10)	16 , 12 (14)	18 , 19 (19)	5 , 7 (6)
	50	126 , 110 (118)	13 , 8 (11)	16 , 13 (15)	21 , 16 (19)	5 , 8 (7)
	150	110 , 114 (112)	13 , 7 (10)	15 , 15 (15)	27 , 21 (24)	9 , 11 (10)
	500	120 , 110 (115)	7 , 5 (6)	23 , 16 (20)	22 , 25 (24)	12 , 14 (13)
	1500	116 , 118 (117)	7 , 12 (10)	13 , 14 (14)	27 , 21 (24)	9 , 10 (10)
	5000	106 , 115 (111)	9 , 8 (9)	23 , 24 (24)	16 , 21 (19)	6 , 7 (7)
		AF-2	NaN_3	AF-2	AF-2	9-AA
Positive control	Concentration (μg/plate)	0.01	0.5	0.01	0.1	80
	Rev./plate (Mean)	557 , 622 (590)	267 , 369 (318)	54 , 76 (65)	275 , 287 (281)	187 , 151 (169)

a : Water for injection (Japanese pharmacopoeia)

AF-2 : 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide

NaN_3 : Sodium azide

9-AA : 9-Aminoacridine hydrochloride hydrate

Rev. : Revertants

Table 2 Reverse mutation test of in *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 and *Escherichia coli* WP2*uvr* A with metabolic activation (dose-finding test) (SR09242)

Compound	Concentration (μg/plate)	Revertants per plate (Mean)				
		S9 (+)				
		TA100	TA1535	WP2*uvr* A	TA98	TA1537
Control [a]		144 , 162 119 (142)	12 , 14 5 (10)	27 , 19 26 (24)	30 , 43 26 (33)	7 , 13 12 (11)
	5	171 , 156 (164)	9 , 10 (10)	15 , 13 (14)	31 , 35 (33)	17 , 21 (19)
	15	133 , 158 (146)	12 , 11 (12)	13 , 10 (12)	39 , 49 (44)	15 , 17 (16)
	50	177 , 138 (158)	13 , 13 (13)	18 , 18 (18)	36 , 31 (34)	8 , 14 (11)
	150	141 , 156 (149)	8 , 10 (9)	13 , 13 (13)	43 , 29 (36)	13 , 9 (11)
	500	130 , 143 (137)	10 , 6 (8)	18 , 12 (15)	24 , 32 (28)	15 , 16 (16)
	1500	143 , 153 (148)	10 , 7 (9)	14 , 14 (14)	24 , 37 (31)	5 , 16 (11)
	5000	136 , 131 (134)	7 , 13 (10)	21 , 27 (24)	36 , 31 (34)	15 , 9 (12)
		2-AA	2-AA	2-AA	2-AA	2-AA
Positive control	Concentration (μg/plate)	1	2	10	0.5	2
	Rev./plate (Mean)	1395 , 1321 (1358)	603 , 650 (627)	1161 , 1244 (1203)	143 , 141 (142)	386 , 345 (366)

a : Water for injection (Japanese pharmacopoeia)

2-AA : 2-Aminoanthracene

Rev. : Revertants

Table 3 Reverse mutation test of in *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 and *Escherichia coli* WP2*uvr* A without metabolic activation (main test) (SR09242)

Compound	Concentration (μg/plate)	Revertants per plate (Mean)				
		S9(−)				
		TA100	TA1535	WP2*uvr* A	TA98	TA1537
Control [a]		140 , 129 108 (126)	7 , 7 10 (8)	18 , 13 13 (15)	12 , 19 15 (15)	9 , 11 15 (12)
	78.1	123 , 121 (122)	6 , 11 (9)	16 , 22 (19)	13 , 17 (15)	9 , 10 (10)
	156	132 , 102 (117)	13 , 11 (12)	13 , 18 (16)	17 , 25 (21)	6 , 7 (7)
	313	131 , 140 (136)	15 , 8 (12)	12 , 11 (12)	16 , 18 (17)	12 , 11 (12)
	625	132 , 127 (130)	11 , 2 (7)	17 , 8 (13)	17 , 6 (12)	8 , 6 (7)
	1250	114 , 123 (119)	8 , 13 (11)	11 , 18 (15)	16 , 15 (16)	9 , 8 (9)
	2500	128 , 123 (126)	3 , 8 (6)	12 , 9 (11)	17 , 18 (18)	9 , 9 (9)
	5000	112 , 141 (127)	4 , 3 (4)	20 , 12 (16)	14 , 23 (19)	9 , 11 (10)
Positive control		AF-2	NaN_3	AF-2	AF-2	9-AA
	Concentration (μg/plate)	0.01	0.5	0.01	0.1	80
	Rev./plate (Mean)	599 , 618 (609)	245 , 265 (255)	70 , 60 (65)	266 , 305 (286)	193 , 334 (264)

a : Water for injection (Japanese pharmacopoeia)

AF-2 : 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide

NaN_3 : Sodium azide

9-AA : 9-Aminoacridine hydrochloride hydrate

Rev. : Revertants

Table 4 Reverse mutation test of [in *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 and *Escherichia coli* WP2*uvr* A with metabolic activation (main test) (SR09242)

Compound	Concentration (μg/plate)	Revertants per plate (Mean)				
		S9 (+)				
		TA100	TA1535	WP2*uvr* A	TA98	TA1537
Control [a]		134 , 171 159 (155)	8 , 9 16 (11)	15 , 20 18 (18)	35 , 27 35 (32)	13 , 8 10 (10)
	78.1	134 , 167 (151)	12 , 13 (13)	12 , 23 (18)	20 , 22 (21)	13 , 6 (10)
	156	164 , 158 (161)	6 , 14 (10)	6 , 19 (13)	20 , 34 (27)	13 , 10 (12)
	313	146 , 191 (169)	9 , 11 (10)	25 , 21 (23)	20 , 36 (28)	7 , 12 (10)
	625	167 , 141 (154)	12 , 11 (12)	14 , 18 (16)	26 , 32 (29)	9 , 15 (12)
	1250	145 , 137 (141)	12 , 8 (10)	14 , 18 (16)	31 , 25 (28)	12 , 6 (9)
	2500	124 , 149 (137)	8 , 12 (10)	12 , 18 (15)	29 , 22 (26)	6 , 10 (8)
	5000	125 , 160 (143)	9 , 8 (9)	13 , 16 (15)	18 , 31 (25)	7 , 14 (11)
Positive control		2-AA	2-AA	2-AA	2-AA	2-AA
	Concentration (μg/plate)	1	2	10	0.5	2
	Rev./plate (Mean)	1481 , 1447 (1464)	545 , 613 (579)	1280 , 1365 (1323)	152 , 159 (156)	415 , 471 (443)

a : Water for injection (Japanese pharmacopoeia)

2-AA : 2-Aminoanthracene

Rev. : Revertants

Table 5 Reverse mutation test of in *Salmonella typhimurium* TA98 without metabolic activation (confirmatory test) (SR09242)

Compound	Concentration (μg/plate)	Revertants per plate (Mean)
		S9 (−)
		TA98
Control [a]		26 , 18 14 (19)
	78.1	18 , 17 (18)
	156	20 , 18 (19)
	313	17 , 27 (22)
	625	16 , 22 (19)
	1250	22 , 18 (20)
	2500	15 , 19 (17)
	5000	12 , 16 (14)
		AF-2
Positive control	Concentration (μg/plate)	0.1
	Rev./plate (Mean)	320 , 350 (335)

a : Water for injection (Japanese pharmacopoeia)
AF-2 : 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide
Rev. : Revertants

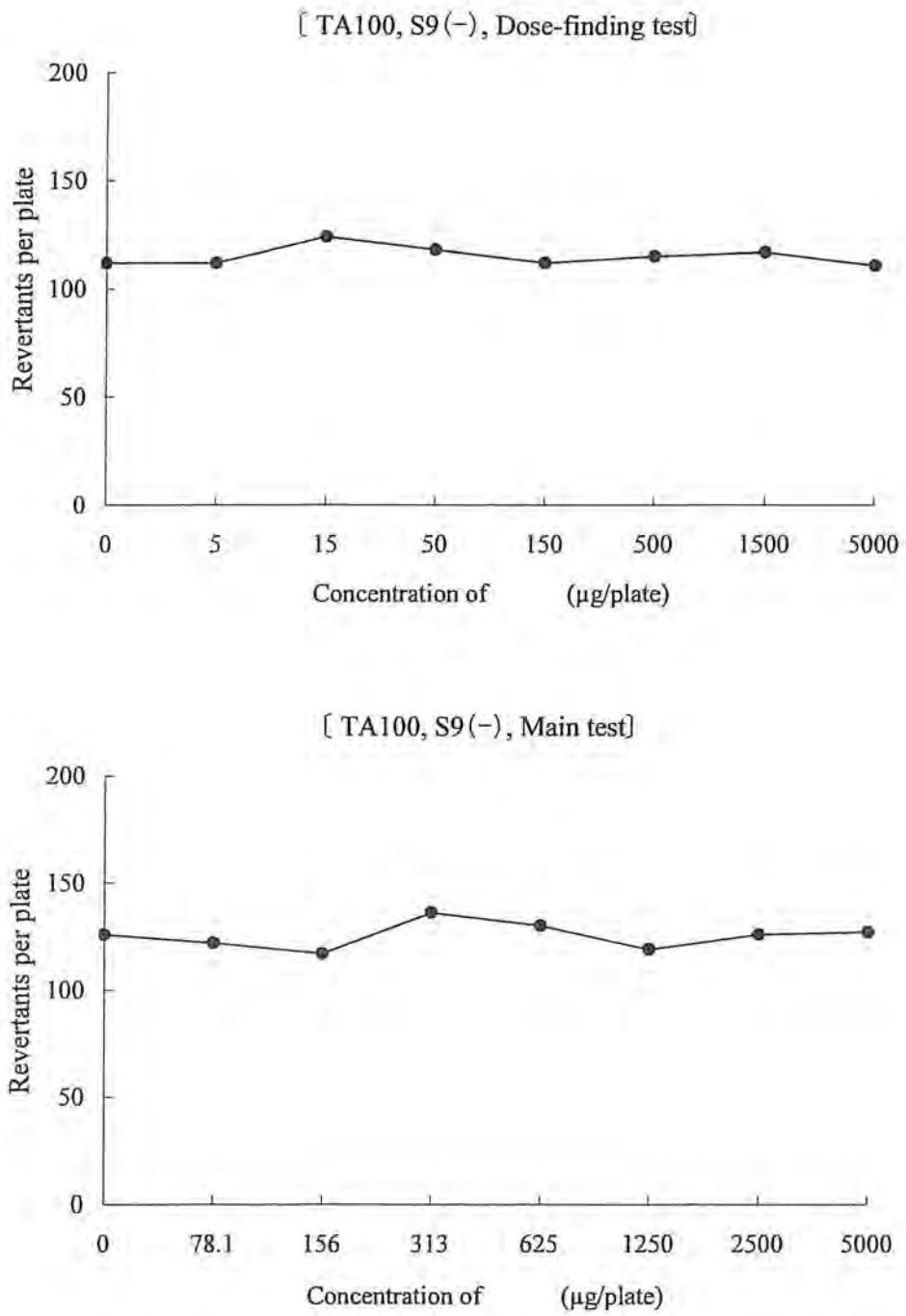
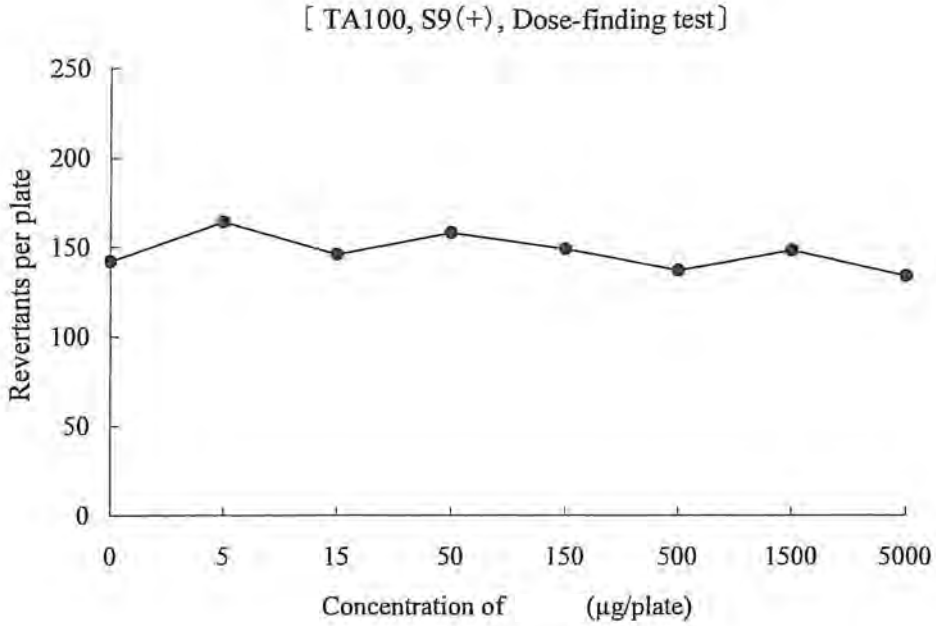


Figure 1-1 Reverse mutation test of in *Salmonella typhimurium* TA100 without metabolic activation (dose-response curves) (SR09242)



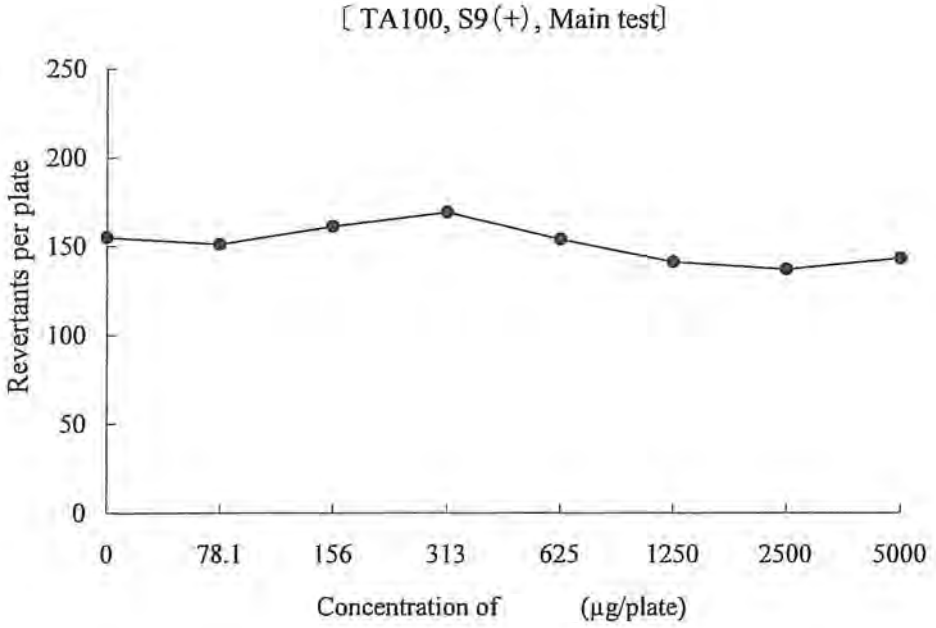


Figure 1-2 Reverse mutation test of in *Salmonella typhimurium* TA100 with metabolic activation (dose-response curves) (SR09242)

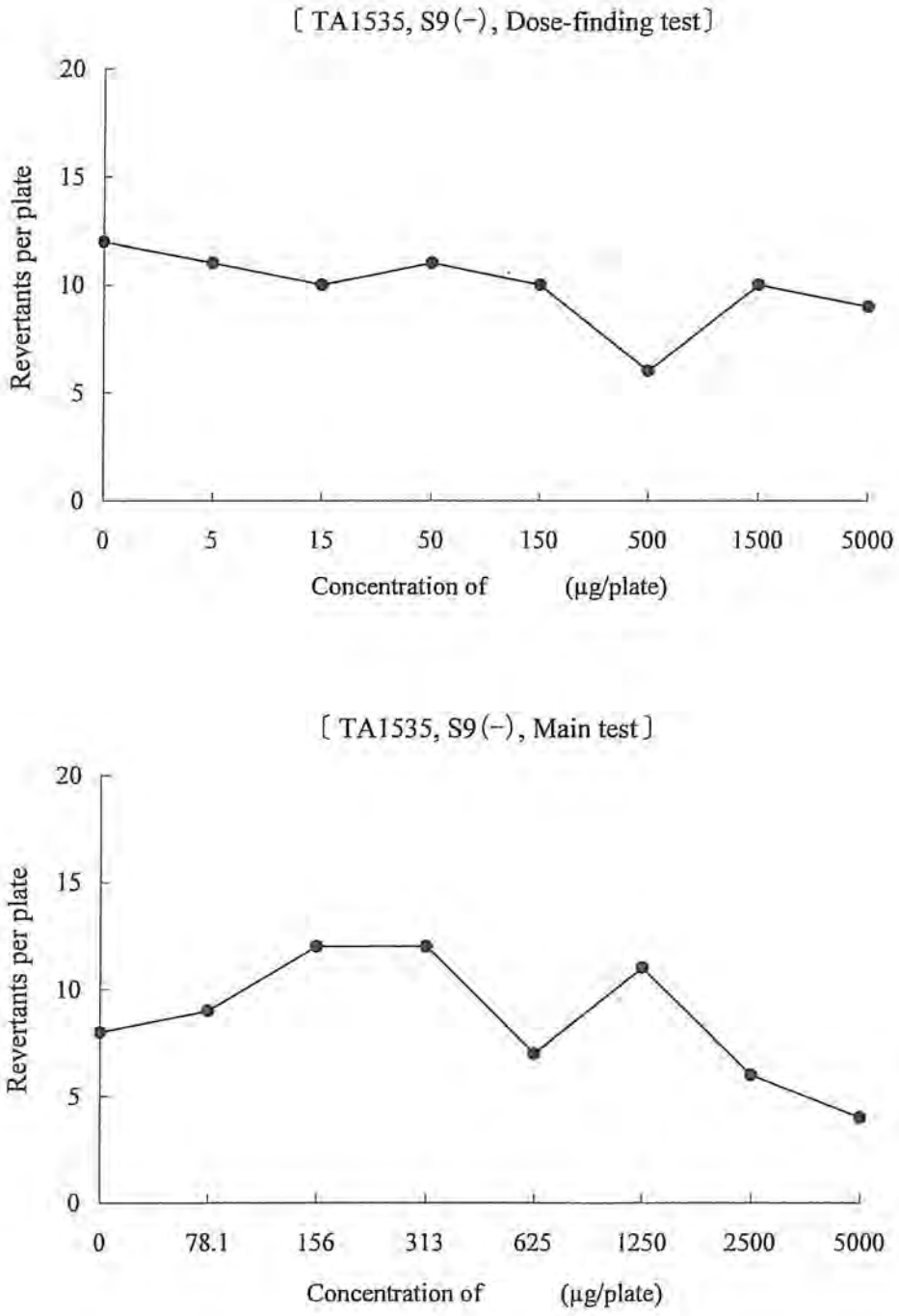
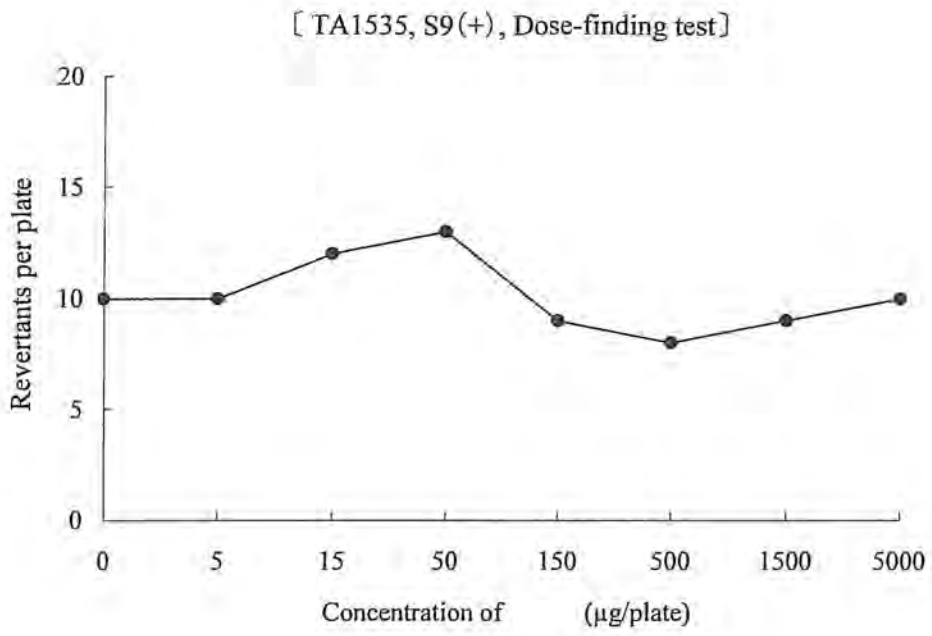


Figure 2-1 Reverse mutation test of in *Salmonella typhimurium* TA1535 without metabolic activation (dose-response curves) (SR09242)



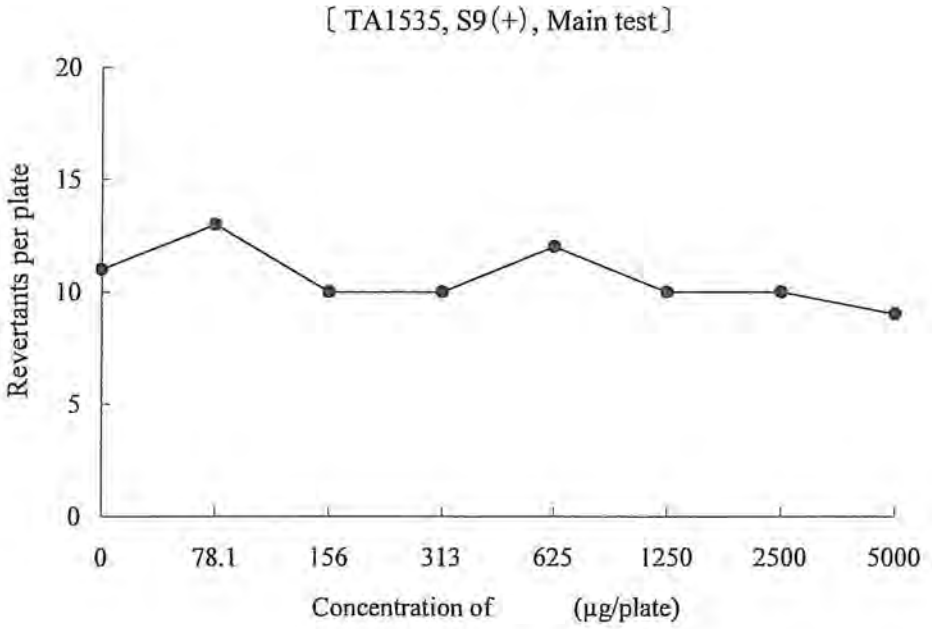


Figure 2-2 Reverse mutation test of in *Salmonella typhimurium* TA1535 with metabolic activation (dose-response curves) (SR09242)

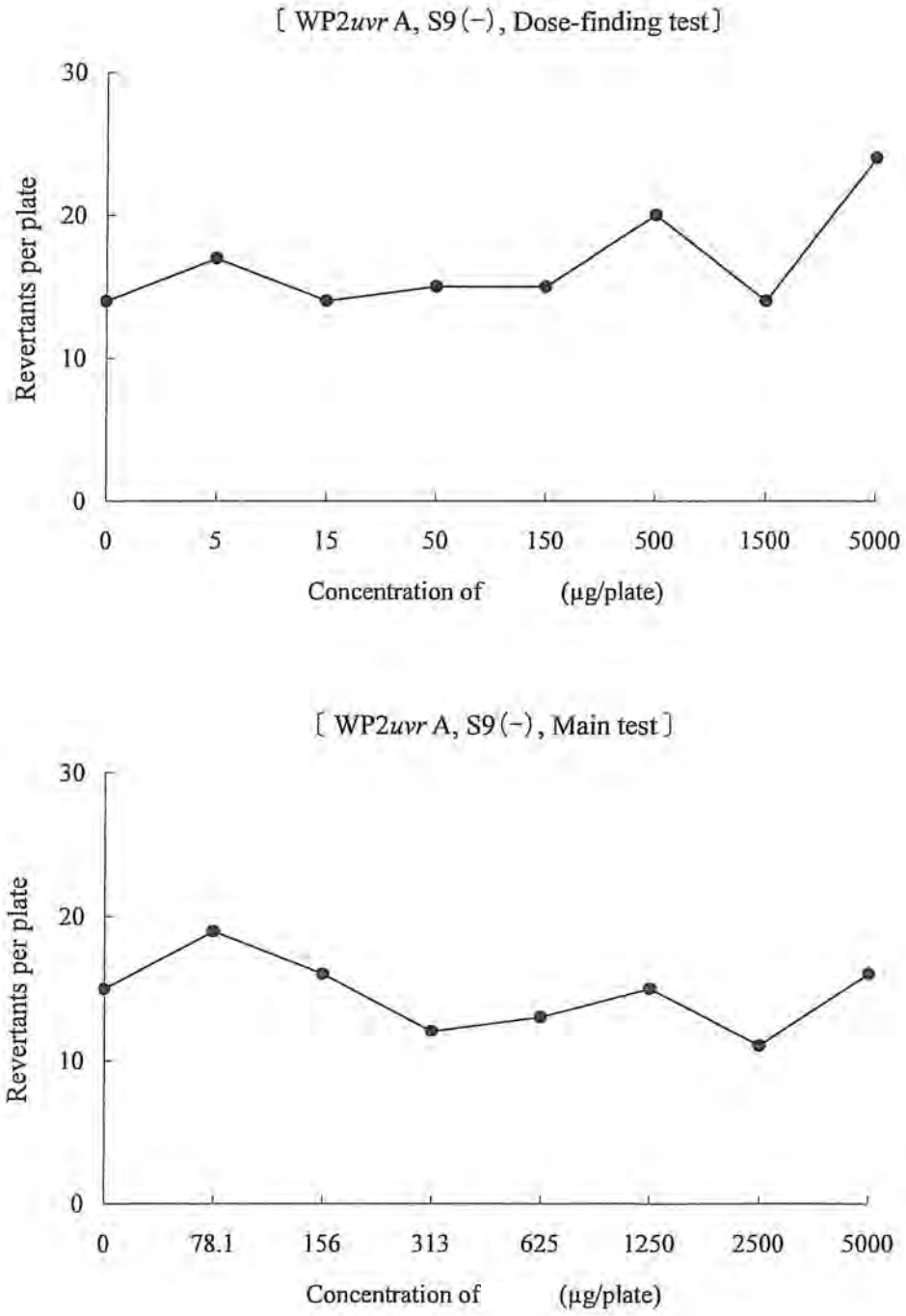
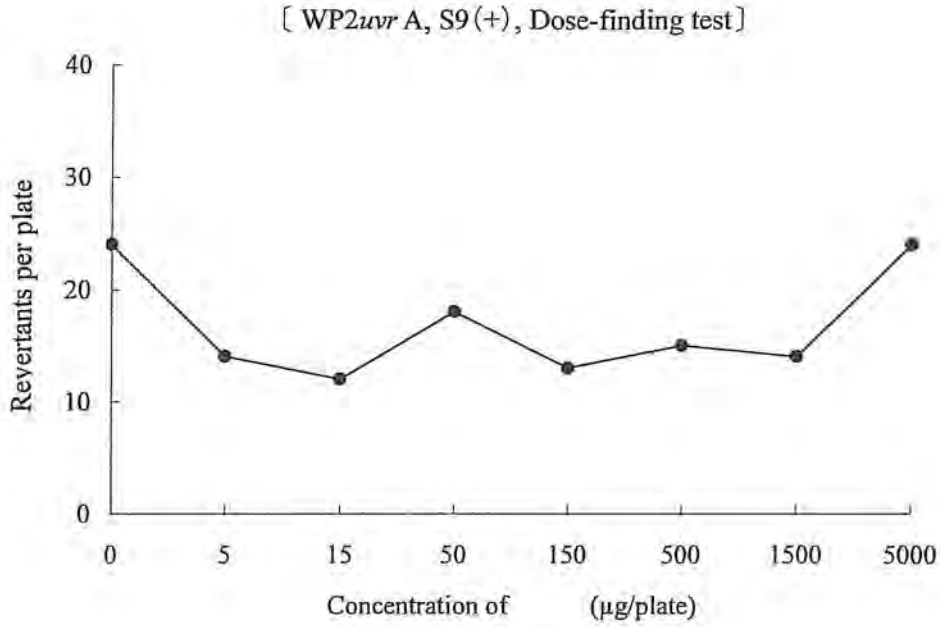


Figure 3-1 Reverse mutation test of in *Escherichia coli* WP2*uvr* A without metabolic activation (dose-response curves) (SR09242)



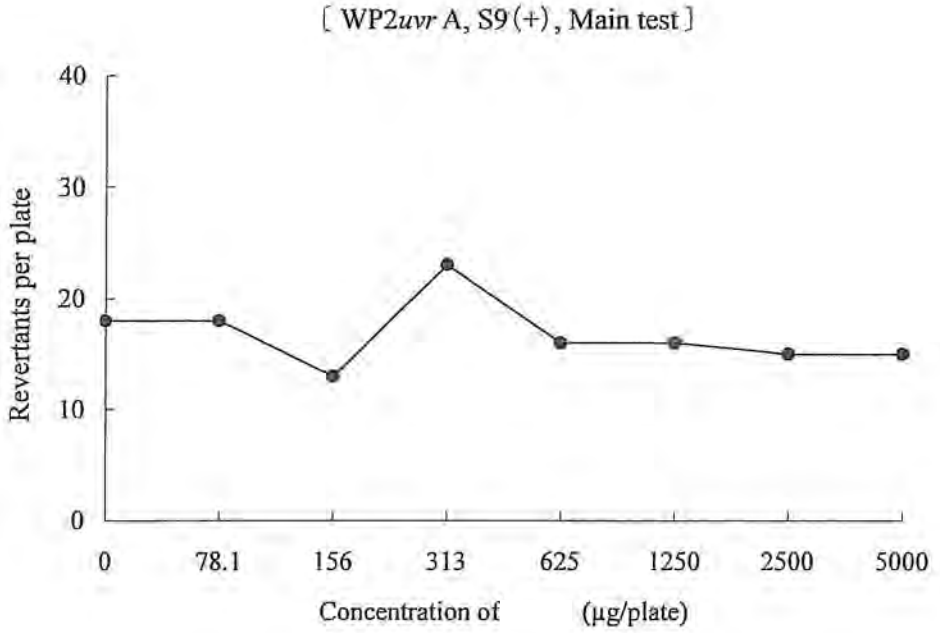
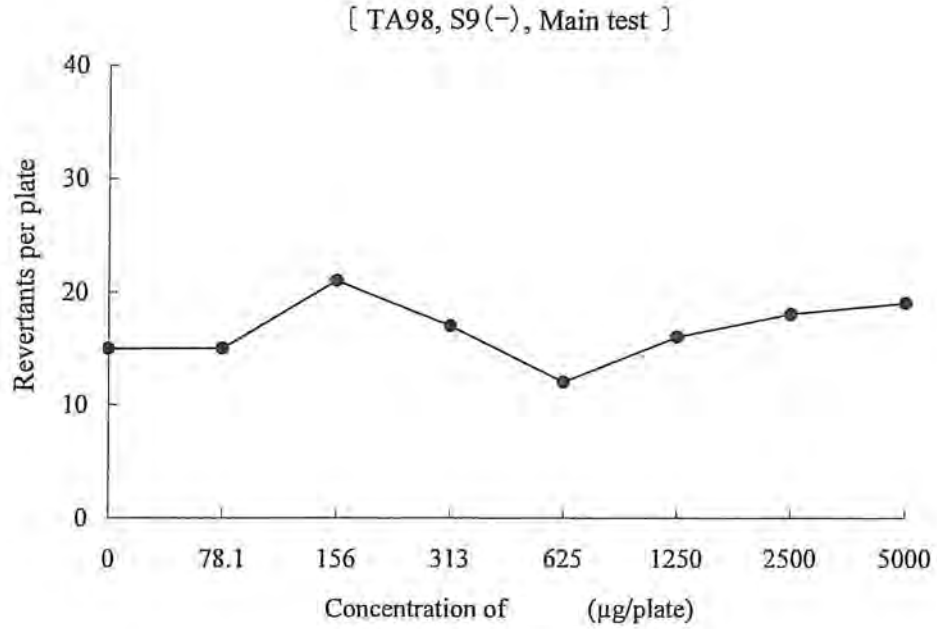


Figure 3-2 Reverse mutation test of in *Escherichia coli* WP2*uvr* A with metabolic activation (dose-response curves) (SR09242)



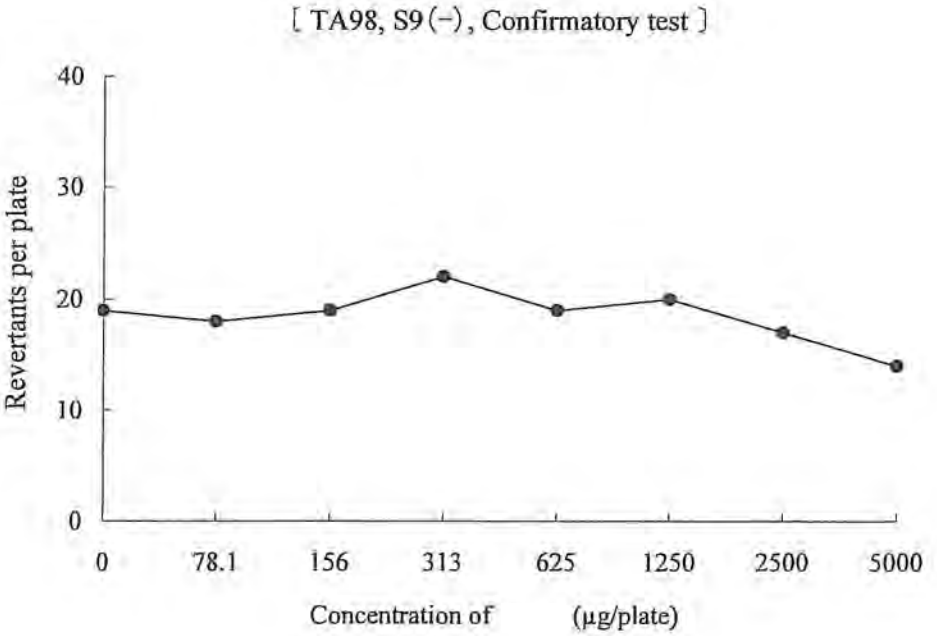
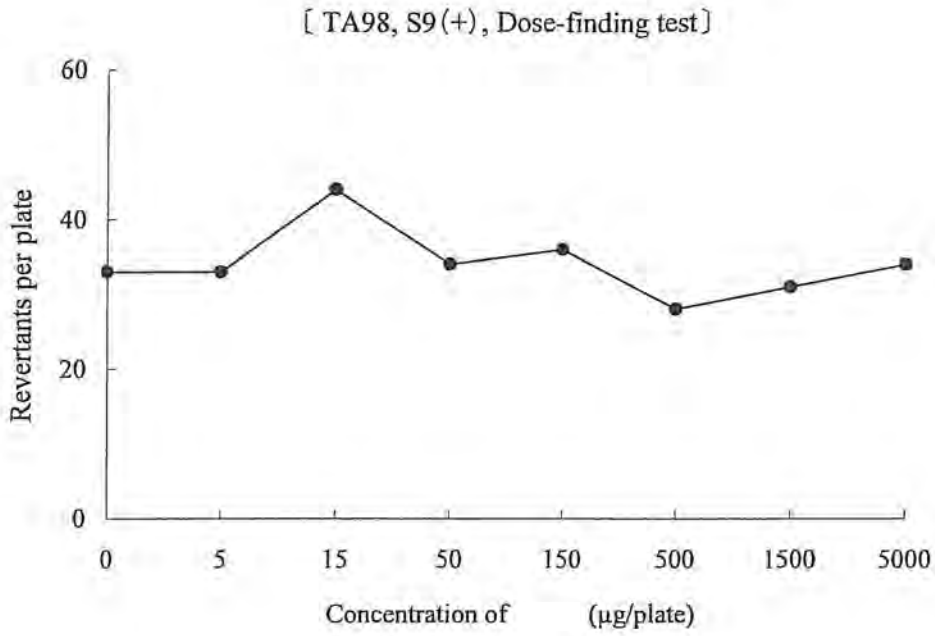


Figure 4-1 Reverse mutation test of in *Salmonella typhimurium* TA98 without metabolic activation (dose-response curves) (SR09242)



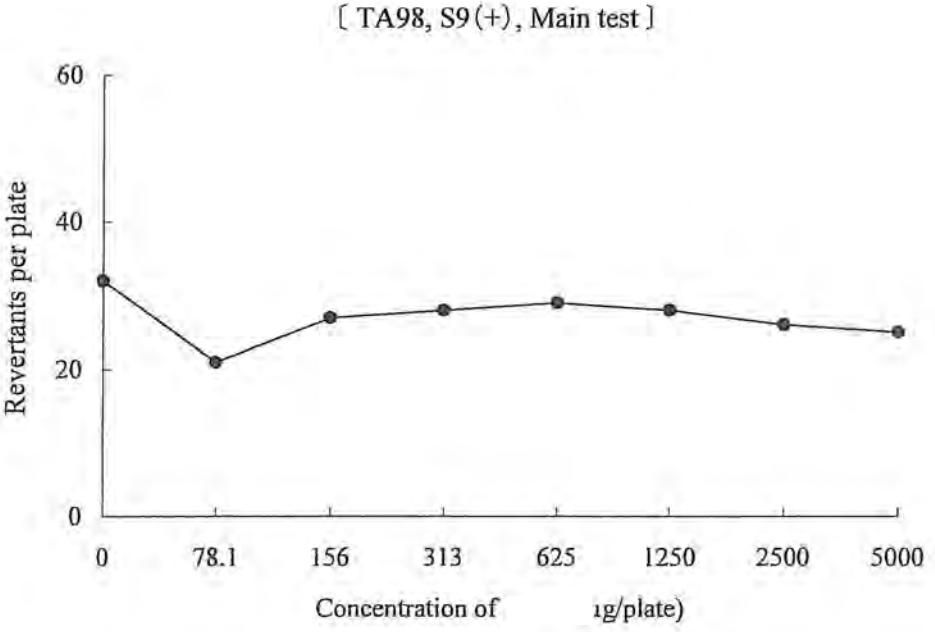
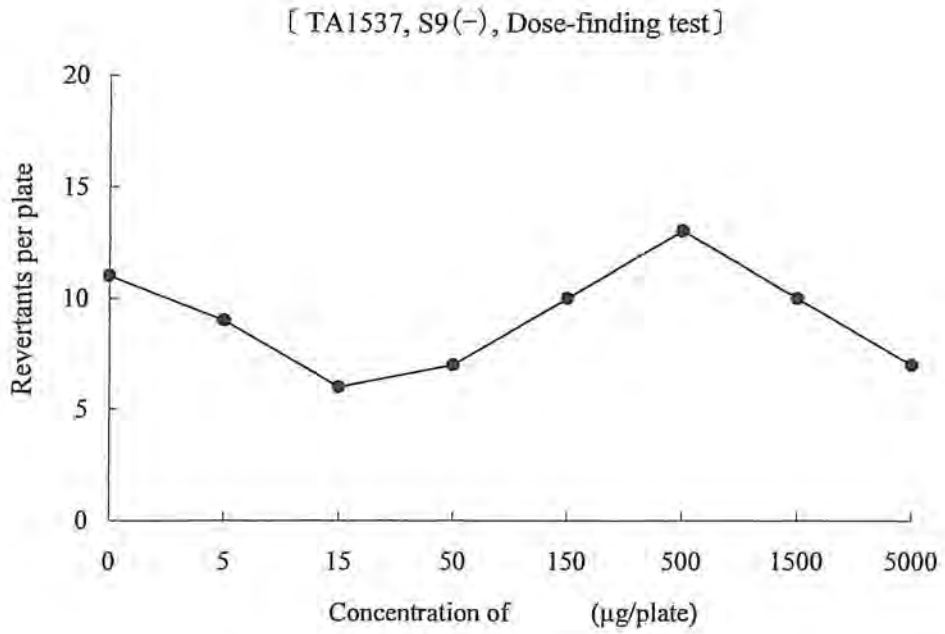


Figure 4-2 Reverse mutation test of in *Salmonella typhimurium* TA98 with metabolic activation (dose-response curves) (SR09242)



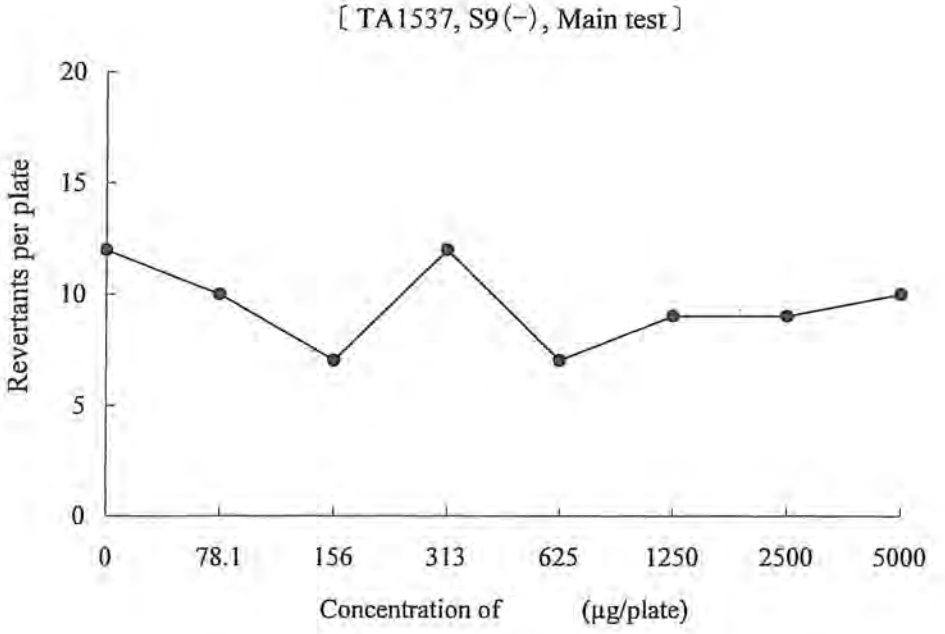


Figure 5-1 Reverse mutation test of in *Salmonella typhimurium* TA1537 without metabolic activation (dose-response curves) (SR09242)

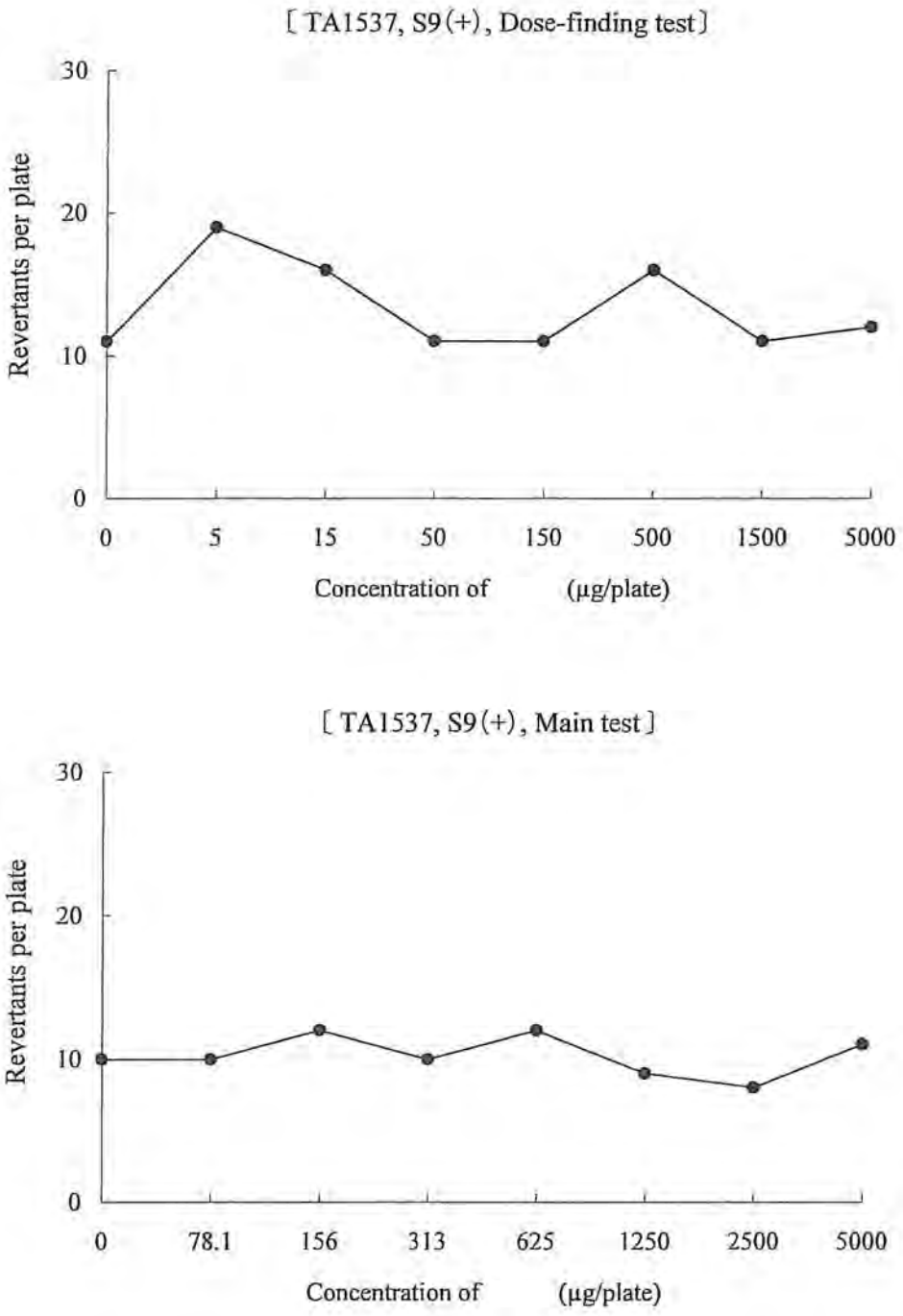


Figure 5-2 Reverse mutation test of in *Salmonella typhimurium* TA1537 with metabolic activation (dose-response curves) (SR09242)

January 14, 2010

Analysis table

1. Name of article

2. Analysis values

Items	Analysis values	Notes

August 1, 2010

Analysis table

1. Name of article

2. Analysis values

Historical control data for reverse mutation test (2010. 5)

Control data (Date : 2009.6~2010.4)

Strain	TA100		TA1535		WP2*uvr* A		TA98		TA1537	
Metabolic activation	S9(−)	S9(+)	S9(−)	S9(+)	S9(−)	S9(+)	S9(−)	S9(+)	S9(−)	S9(+)
No. of data	n = 47	n = 95	n = 39	n = 39	n = 38	n = 38	n = 40	n = 39	n = 38	n = 38
Mean ± S.D.	105 ± 11	131 ± 13	10 ± 4	11 ± 3	19 ± 5	20 ± 4	18 ± 4	31 ± 5	17 ± 6	18 ± 7
Maximum	128	169	24	22	30	32	27	40	29	33
Minimum	75	100	5	6	9	12	12	21	5	8
X-R-Rs [a]	73 ~ 137	96 ~ 166	2 ~ 18	3 ~ 19	3 ~ 35	7 ~ 33	7 ~ 29	15 ~ 47	0 ~ 36	2 ~ 34
Reference : Control data (Date : 1992.2~2010.4)										
No. of data	n = 704	n = 741	n = 600	n = 594	n = 585	n = 583	n = 706	n = 693	n = 600	n = 596
Mean ± S.D.	123 ± 19	136 ± 18	10 ± 2	11 ± 3	18 ± 4	21 ± 4	17 ± 4	29 ± 5	12 ± 4	16 ± 5
Maximum	248	219	24	39	44	45	39	45	29	39
Minimum	75	82	5	6	7	11	7	10	4	2
X-R-Rs [a]	86 ~ 160	96 ~ 176	5 ~ 15	6 ~ 16	7 ~ 29	10 ~ 32	6 ~ 28	16 ~ 42	4 ~ 20	5 ~ 27

Positive control data (Date : 2009.6~2010.4)

Strain	TA100		TA1535		WP2*uvr* A		TA98		TA1537	
Metabolic activation	S9(−)	S9(+)	S9(−)	S9(+)	S9(−)	S9(+)	S9(−)	S9(+)	S9(−)	S9(+)
Compound	AF-2	2-AA	NaN_3	2-AA	AF-2	2-AA	AF-2	2-AA	9-AA	2-AA
Concentration (μg/plate)	0.01	1	0.5	2	0.01	10	0.1	0.5	80	2
No. of data	n = 47	n = 95	n = 39	n = 39	n = 38	n = 38	n = 40	n = 39	n = 38	n = 38
Mean ± S.D.	644 ± 92	1643 ± 307	300 ± 47	546 ± 61	81 ± 17	1214 ± 127	348 ± 75	209 ± 60	222 ± 52	469 ± 108
Maximum	882	2291	482	650	123	1424	510	317	356	695
Minimum	505	1006	214	346	53	851	184	122	89	263
X-R-Rs [a]	490 ~ 798	1034 ~ 2252	191 ~ 409	386 ~ 706	60 ~ 102	924 ~ 1504	226 ~ 470	148 ~ 270	68 ~ 376	192 ~ 746
Reference : Positive control data (Date : S9(-) ; 1998.4~2010.4, S9(+) ; 1992.2~2010.4)										
No. of data	n = 527	n = 624	n = 495	n = 542	n = 494	n = 533	n = 529	n = 559	n = 498	n = 541
Mean ± S.D.	788 ± 155	1299 ± 341	263 ± 51	303 ± 106	124 ± 46	880 ± 224	360 ± 87	269 ± 68	273 ± 71	273 ± 109
Maximum	1352	2501	482	686	454	1765	594	607	640	791
Minimum	505	227	132	105	53	309	180	116	89	73
X-R-Rs [a]	543 ~ 1033	796 ~ 1802	165 ~ 361	178 ~ 428	76 ~ 172	598 ~ 1162	211 ~ 509	155 ~ 383	116 ~ 430	137 ~ 409

[a] X-R-Rs =X±2.66Rs

X =Mean

Rs =Mean of $(X_i - X_{i-1})$